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OPTIMIZATION OF PHYTASE PRODUCTION BY *Enterobacter cloacae* ISOLATED FROM LEGUME RHIZOSPHERE

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Running title: Phytase production by *Enterobacter cloacae*

ABSTRACT

Phytase producing bacteria (PPB) are utilized as plant growth promoting rhizobacteria (PGPR) to improve plant growth. Phytase is an enzyme that can hydrolyze the phosphoester bond in organic phosphorous (phytic acid), forming ester phosphate and inorganic phosphate, allowing for the availability of phosphorous to plants. The aim of the current study is to isolate the PPB from *Vigna sinensis* rhizosphere and optimize physicochemical conditioning of PPB. The screening of their ability to hydrolyze organic phosphates (calcium phytate) was performed in solid and liquid phytase screening medium (PSM). The results of these isolation studies indicated that a total of 13 bacteria were positive for this enzyme's production. This was indicated by clear zones of hydrolysis that were observed around the colony. Based on the screens that were conducted, *Enterobacter cloacae* strain B1 was found as the largest hydrolysis efficient bacteria (3.43) on solid medium. The Phytase-production of strain *Enterobacter cloacae*, which was grown in liquid PSM, showed 0.92 U/ml after 48 hours of incubation. This strain produced optimum levels of phytase in the presence of lactose and monoammonium phosphate ($NH_4H_2PO_4$), which served as carbon and nitrogen sources respectively. The optimum results were obtained at 30^{o}C and a pH of 5.0 within the physical parameters. The PPB obtained in the present study can be exploited for their use as biological fertilizers for plants.

Keywords: Phytase producing bacteria, *Enterobacter cloacae*, phytase

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria commonly found in the rhizosphere, root surface or any other area associated with plant roots. PGPR is able to boost plant growth and protect plants from diseases and abiotic stress through various mechanisms. Several important mechanisms of PGPR are biological nitrogen fixation, ACC deaminase activity, production of siderophores, phytohormones and phosphate dissolution (Grover *et al*. 2011; de Souza *et al*. 2015). In the rhizosphere, microorganisms play very important roles in the transformation and mobilization of micro and macro nutrients in the soil, thus increasing plant growth (Jha *et al*. 2012). Soil phosphorus is an important source of nutrient for plant growth and as well as development among other macro nutrients. However, the availability of natural phosphorus is usually low. It is due to the very slow process of phosphorus solubilization to the available form while, in contrast, the transformation to the insoluble form is fairly rapid (Jorquera *et al*. 2011).

Nearly 30-65% of the total phosphorous (P) in the soil is in its organic P form. Phytate is one of the dominant organic P in the soil and it is not available for plant use. This is due to phytate possessing strong bonds with mono or divalent cations and also due to the fact that it is able to form complexes with other nutrients such as metal ions (Ca, Mg, Fe, Cu) (Cerino *et al*. 2012; Selle *et al*. 2012; Shim & Oh 2012). Some PGPRs are able to dissolve organic or inorganic phosphate, thus it becomes available for plant growth. PPB are members of PGPR that have the ability to hydrolyze phytate by secreting phytase that provides P inorganic esters, allowing P to become available for plants (Greiner *et al*. 2007; Shivange *et al*. 2010; Richardson & Simpson 2011). It is contained in plants, microorganisms and animal tissues. PPB are widely found in agricultural fields, grasslands and forests. There are researchers that have isolated phytase-producing bacteria from various rhizospheres. *Enterobacter, Bukholderia, Pseudomonas* and *Pantoea* have all been isolated from legume plant rhizosphere, white lupin (*Lupinus albus*), and other plant rhizosphere (Yoon *et al*. 1996; Unno *et al*. 2005; Jorquera *et al*. 2008).

The production of phytase is greatly affected by the composition of the medium used for bacterial culture. The medium's physical and nutritional conditions have a significant effect on enzymatic production. The objective of the experiment was to isolate PPB from leguminous plant rhizosphere and to optimize phytase production by *Enterobacterium cloacae* under various physical conditions (incubation time and initial pH, temperature) as well as utilising different sources of nutrients (carbon (C) and nitrogen (N) sources).

MATERIALS AND METHODS

Bacterial isolation

Phytase production bacteria were isolated from soil samples taken from the legume plant rhizosphere around Cibinong, West Java. One gram of soil was dissolved into 9 ml 0.8% sterile NaCl solution and was serially diluted. Around 0.2 ml of the final solution was placed in a sterile Petri dish and then poured with phytase screening medium (PSM) agar (1.5% glucose, 0.5% (NH4) $2SO_4$, 0.01% NaCl, 0.05% KCl, 0.001% $FeSO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 0.001% $MnSO_4$, pH 6.5 with 0.5% calcium phytate) (Kerovuo et al. 1998). The Petri dish was incubated for 7 days at room temperature and evaluated for colonies growing halo zones. Colonies with halo zones around them were further purified with repeated subcultures. The colony and halo zone diameters was measured after 1-7 days of incubation. The halo zone formed surrounding the colony revealed phytate hydrolization and was expressed as Hydrolysis Efficiency. Hydrolysis efficiency (HE) = (Diameter of halo zone - diameter of colony)/Diameter of colony (Dobre *et al*. 2015).

Identification of the selected producing phytase bacteria

There were 13 phytase producing bacteria (PPB) isolates, namely PPB strain B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, and B13 from the legume rhizosphere that demonstrated the ability to hydrolyze Ca phytate (hydrolysis efficiency) in PSM solid. Based on the analysis of phytase production on solid PSM, PBB strain B1 is the highest hydrolysis efficiency of bacteria (34.2), selected for further identification and analysis. The bacterial isolate was identified by following the Otsuka *et al*. (2008) method based on 16S rRNA gene sequences with 16S-9F (5-GAGTTTGATCCTGGCCC-3) and 16S-15 10R (5-GGCTACCTTGTTACGA-3) primary.

Phytase Activity

Bacterial isolates were inoculated into 50 ml of liquid phytase media, then incubated in a rotary shaker (200 rpm) at room temperature (30°C) for 24, 48, 72 and 92 hours. The culture was centrifuged at 10000 g for 10 min at 4° C. The supernatant was extracted as extracellular source of phytase and calcium (Ca) phytate was used as the substrate in phytase activity assessment. The activity of this enzyme was determined by measuring the amount of inorganic phosphate produced. The reactant mixture comprised of 0.5% calcium phytate dissolved in sodium acetate buffer (0.1 M, pH 5.5), and 0.1 ml of the supernatant. After incubation at 45° C for 30 minutes, the reaction was inhibited by adding 5% trichloroacetic acid. Around 160µl of reagent dye consisting of 10N H_2SO_4, 10% ammonium molybdate and 5% $FeSO_4$, was then added. It was allowed to stand for 30 min after incubating at 45 °C. The absorbance was measured by using a spectrophotometer set at 660 nm wavelength. One enzyme unit (IU) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate in 1 minute (Kumar et al. 2013).

Physico-chemical optimization on phytase production (Sreedevi and Reddy, 2012)

Effect of incubation time

To investigate the optimum time for growth and production, 10% of 10^9 cfu mL^{-1} *Enterobacter cloacae* inoculum was inoculated into 100 ml of liquid PSM using a 250 ml Erlenmeyer flask and incubated in a rotary shaker at 120 rpm for 4 days at room temperature. The culture was harvested at an interval of 24, 48, 72 and 96 hours.

Effect of pH

To obtain the optimum pH, *E. cloacae* was grown in various initial pH viz. 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The liquid PSM was adjusted using 1N HCl and 1N NaOH.

Effect of Temperatures

E. cloacae was incubated at different temperatures (30,40 and 50^{o}C) in pH 6.5 to study production levels for 48 hours in liquid PSM.

Effect of N sources

To determine the effect of N sources on production, organic nitrogen (tryptone and beef extract) and inorganic nitrogen ($(NH_4)H_2PO_4$ and $NH_4(NO_3)$) were used to replace $(NH_4)_2SO_4$ in pH 6.5 at 30^{o}C in the liquid PSM.

Effect of carbon (C) sources

To determine the effect of carbon on production, the bacteria was inoculated in liquid PSM (pH 6.5 at 30^{o}C and $(NH_4)_2SO_4$ as N source), supplemented with different C sources viz. glucose, dextrose, lactose and maltose.

RESULTS AND DISCUSSION

Bacterial isolation

PPB are members of PGPR that have the ability to hydrolyze phytate by secreting phytase to produce phosphate esters and inorganic phosphorous, allowing P to become available for plant use (Greiner *et al*. 2007; Shivange *et al*. 2010; Richardson & Simpson 2011). The activities of all bacteria were assayed using PSM agar, and this was indicated by the formation of clear zones around the colony.

There were 13 bacterial isolates from the legume rhizosphere that demonstrated the ability to hydrolyze Ca phytate in PSM solid (Fig.1). The hydrolysis efficiency, based on halo zone and colony diameter, ranged from 0.56 to 3.43 (Fig. 2). Sreedevi & Reddy (2012) stated in their results that hydrolysis efficiency from 54 PPB that were isolated from rhizosphere soil, (cattle shed soil and poultry farm soil) ranged from 4 to 200%. Some researchers have isolated the PPB from various plant rhizospheres around legumes (*Lulinus albus* (L.) (Unno *et al*. 2005; Acuna *et al*. 2011). Similarly, Jorquera *et al.* (2008) had isolated bacteria from various plants rhizosphere grown in volcanic soils, such as wheat (*Triticum aestivum*), oats (*Avena sativa*), lupin (*Lupineus luteus*), *Lolium perenne* and *Trifolium repens*, which have the ability to use sodium (Na) phytate and Ca phosphate on agar media.

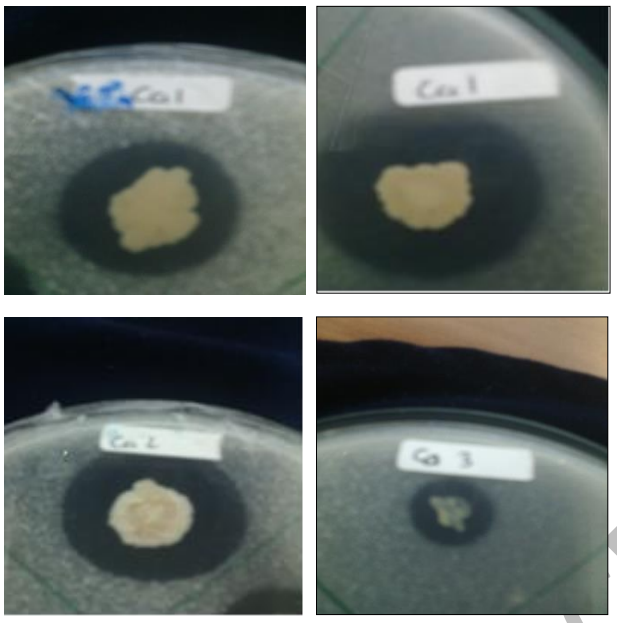

Figure 1 Halo zone around Phytase Producing Bacterial (PPB) colony on solid PSM

Note: Photo of colonies were taken from one petri dish which was divided into 4 sections, each photo has been taken from a different petri dish.

1. Ca 1 (Isolate B1); 2. Ca 1 (Isolate B1); 3. Ca 2 (Isolate B2); and 4. Ca 3 (Isolate B3)

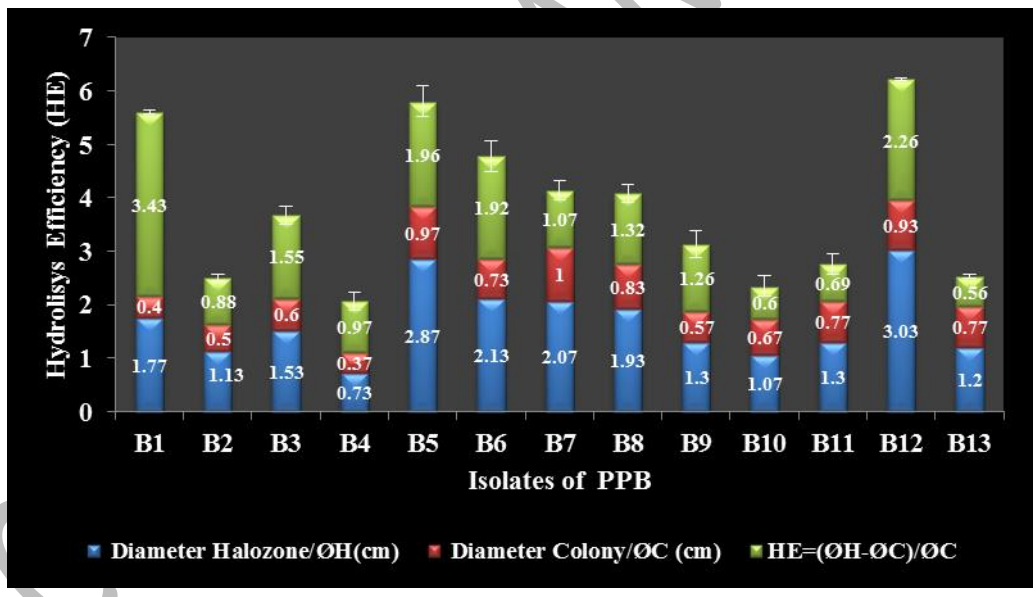

Figure 2 Hydrolysis efficiency ability of the isolates

Note: Value represent mean ± standard deviation (*n*=3)

Bacterial Identification

One isolate (B1) that produced the highest hydrolysis efficiency (3.43) was identified as *Enterobacter cloacae.* Partial sequences of 16S rRNA genes were compared to the database on GeneBank database of NCBI by use of the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed using the neighbor-joining methods of the MEGA 7 program. Sequences from all species of *Enterobacter* genus were referred from the NCBI database. The

corresponding GeneBank accession numbers were labeled after the name of the species and strains. Associated taxa were clustered in the 1000 replicates from the boostrap test and the substitution model used Junkes-Cantor model with gamma (1). *Aquifex pyrophilus* Kol5a was used as the outgroup taxon to determine the root of the tree. With a boostrap value of 56% to the *Enterobacter cloacae* subsp. and the B1 (B184a) sequence being found in the *Enterobacter cloacae* group, this suggested that it had members within the *Enterobacter* genus and was similar with *Enterobacter cloacae* (Fig.3).

Figure 3 The phylogenetic tree of B1 isolates

Physico-chemical optimization on phytase production

Effect of incubation time

Fig.4. shows the effect of incubation time on phyatse activity. Correspondingly, quantitative assesment on liquid PSM showed that *Enterobacter cloacae* bacteria was able to produce phytase of 0.92 U / ml. Similarly, Gulati (2007) reported that *Bacillus laevolacticus* (Tj1, Tj, 3 Tj4, Tj6)

strains isolated from legume rhizosphere yielded as much as 0.158, 0.216, 0.202, and 0.283 U / ml, respectively. Fu *et al*. (2011) discovered that *Bacillus licheniformis* grown in shakers under optimum conditions resulted in high activity (0.276 U/ml). Sasirekha *et al*. (2012) reported that *P aeruginosa* isolated from rhizosphere soil samples showed an activity of 22.165 U/ml. Comparably, Gui E Li *et al*. (2013); Tungala *et al*. (2013) reported the presence of activity produced by bacteria grown on PSM media containing Na phytate both qualitatively and quantitatively, was about of 2.24-2.58 U / ml and 12.85U / ml respectively.

Phytase activity was observed after a 24-hour incubation period and a significantly high level of enzyme activity (0.92 U/ml) was obtained during 48 hours of incubation, which then decreased at 72 and 92 hours. Incubation time plays an important role in maximum enzyme production. The production period was different from one bacteria to another. The result of the study showed that production started 24 hours after incubation and increased to optimum levels after 48 hours of incubation. This result is in conformity with Kasli *et al*. (2016) who reported that the maksimum phytase production of *Enterobacter cloacae* strain PSB 45 was found at 48 hours of incubation. Muslim *et al*. (2018) revealed the result of the research that the optimum production of *E. aerogenes* at 48 hours. Shamna *et al*. (2012) demonstrated that a stationary growth phase occurred around 48 hours (109 U/ ml) and phytase production occurred after 36 hours of cultivation. Ogbonna *et al*. (2017) observed that the maximum activity of *Pseudomonas aeruginosa* and *Aspergillus niger* were found at 24 hours and 48 hours of incubation respectively. Trivedi *et al.* (2017) reported that optimum production level occurred after 44 hours of incubation while Yoon *et al*. (1996) showed that the maximum production of *Enterobacter* sp4. was obtained after 72 hours of incubation. Time variations depended on nutrient availability in the medium and bacterial culture conditions. The surrounding environmental parameters also affected bacterial cultivation time.

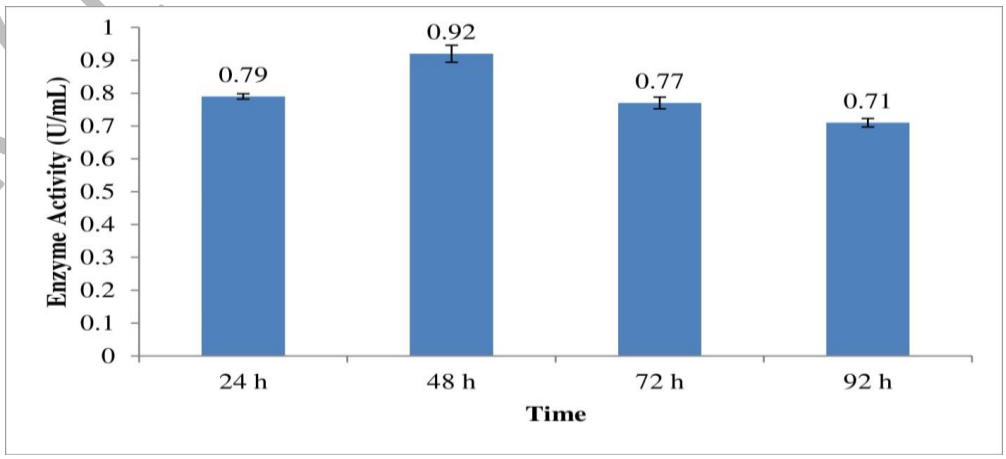

Figure 4 Effect of incubation time (pH 6.5 at 30^{o}C) on phytase production by *Enterobacter cloacae*

Effect of pH

Phytase production was studied at various pH, ranging from pH 3.0 to pH 8.0. In this study, the optimum pH for *Enterobacter cloacae* to produce 0.92 U/ml was pH 5 (Fig.5). The pH of cultivation media has a significant role in the production of phytase by bacterial strain, whereas pH impacts extracellular enzyme activity and the metabolism of microorganisms directly (Moreira *et al*. 2014; Farouk, 2015). In accordance to this result, Tang *et al*. (2010), reported that the highest production by lactic acid-producing bacteria was obtained at pH 5.0. Correspondingly, Farouk *et al*. (2015) stated that the optimum activity by bacterial strain BAFA faifi 103, BAFA faifi 11 and BAFA faifi 117 occurred at the pH of 5.0. Selvamohan *et al*. (2012) also reported that *Pseudomonas* sp generated the highest activity at pH 5.00. Jorquera *et al*. (2017) obtained that the optimum pH to produce phytase for two isolates (9B and 15C) were pH 5.0. While, *Enterobacter* sp.4, *E. intermedius* PHY03, *E. cloacae* PSB45 and *E. aerogenes* produced the maksimum phytase at pH 5.5, 6.5, 7.0 and 5.5 respectively (Yoon *et al*. 1996; Aziz *et al*. 2015; Kasli *et al*. 2016, Muslim *et al*. 2018)

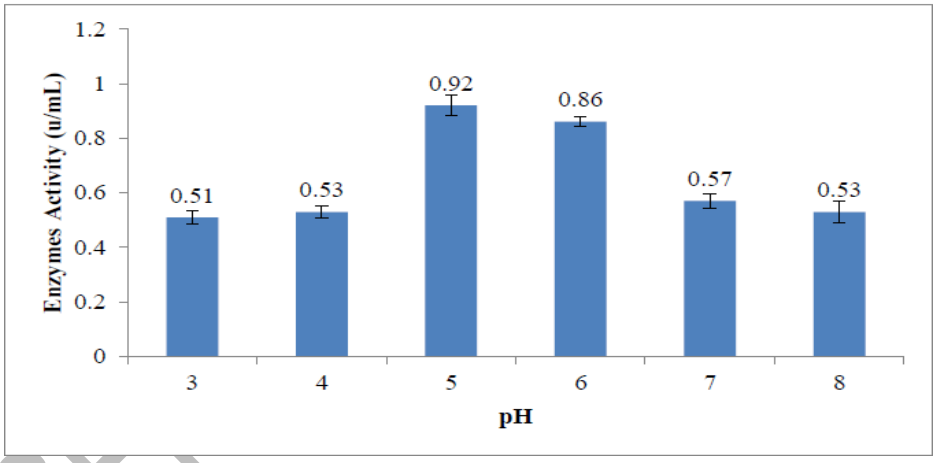

Figure 5 Effect of pH on phytase production by *Enterobacter cloacae*

Effect of Temperature

Apart from pH, temperature is also an essential factor for detecting activity. The highest phytase production of *Enterobacter cloacae* was observed at the incubation temperature of 30^{o}C (0.89 U/ml) (Fig.6). When temperature was increased, there was a noticeable decrease in enzyme production. The result of Ebrahimian *et al*. (2017) revealed that optimized condition of temperature for production by *Citrobacter farmer* strain phas32 was 30^{o}C. Saribuga *et al*. (2014 showed that the optimum enzyme activity of *L. plantarum* also occurred at a temperature of 30°C. The highest production of *Rhizopus oligosporus* (Gautam *et al*. 2002), *Aspergillus ficuum* TUB F-1165 (Gunashree & Venkateswaran 2008), and *Aspergillus niger* (Sandhya *et al*. 2015) occured at 30°C.

While, Yoon *et al*. (1996); Kim *et al*. (2002) discovered that *Enterobacter* spp and *Peudomonas* sp. isolated from legume plant rhizosphere and soil around cattle shed respectively had the highest activity at 37^{o}C. The maximum production from *Pseudomonas* sp. was found at 37^{o} C (Sasirekha *et al*. 2012). Also, Ogbonna *et al*. (2017) showed that the optimum activity by both P. *aeruginosa* and A. *niger* were observed at 37^{o}C. According to Vohra & Satyanarayana (2003), the optimum temperature for production of most microorganisms ranged between 25 to 37°C. On the contrary, Kasli *et al*. (2016) and Muslim *et al*. (2018) reported that the optimum phytase production from *Enterobacter cloacae* and *E. aerogenes* were obtained at 70 and 50^{o}C respectively, higer than in this study.

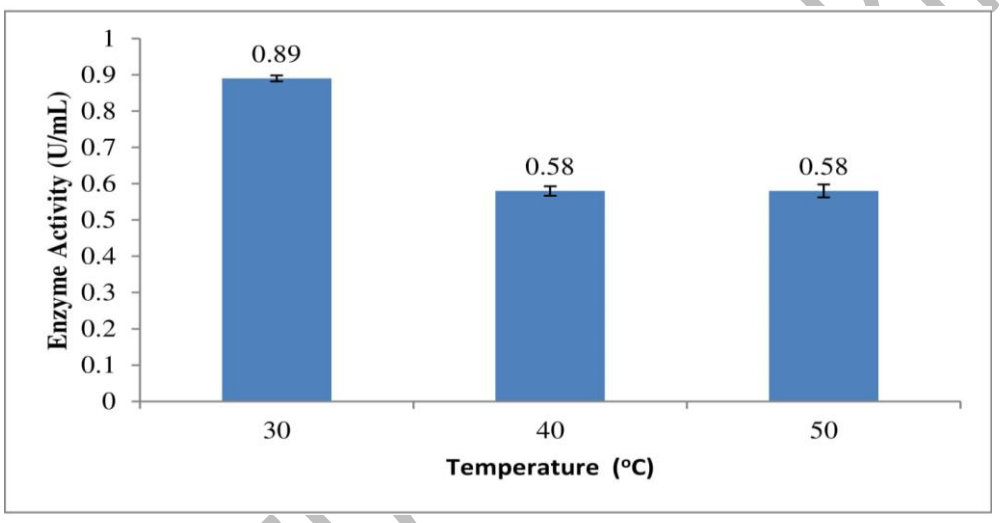

Figure 6 Effect of Temperature on phytase production by *Enterobacter cloacae*

Effect of Carbon sources

The effect of different carbon sources (glucose, dextrose, lactose and maltose) on production was shown in Fig.7. Among different carbon sources studied, the highest yield was obtained with lactose (0.91 U/ml), followed by glucose 0.83 U/ml, dextrose 0.81 U/ml and maltose 0.73 U / ml.

The appropriate type and amount of nutrient sources are important factors that will increase production. The utilization of the best C source in improving activity has been reported by several researchers. In this study, the bacteria *Enterobacter cloacae* showed the highest production when incubated on a media with lactose as C source. In accordance with this result, Aziz *et al.* (2015) reported that *Lactobacillus casei* PHY02 and *Klebsiella pneumonia* PHY30 produced higher activity when grown on media with lactose as the C source, while, *Enterobacter intermedius* PHY03 was suitable in glucose. Similarly, the results of Ogbonna *et al.* (2017) presented that the use of C from lactose in the media produced the highest activity from both P. *aeruginosa* and A.

niger. The results obtained from Demirkan *et al*. (2014) revealed that the maximum phytase activity was found when lactose and wheat brand in the media were used as C sources.

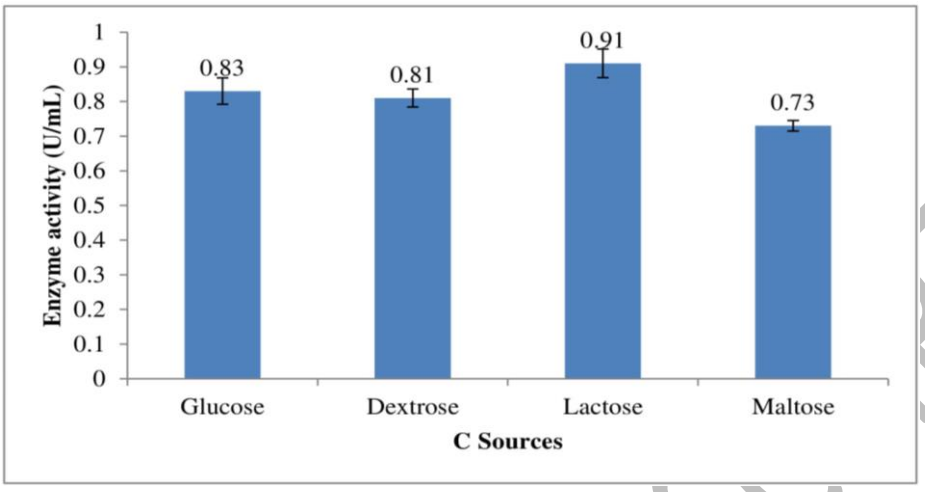

Figure 7 Effect of C sources on phytase production by *Enterobacter cloacae*

Effect of Nitrogen sources

Aside from C source, inorganic nitrogen sources ($(NH_4)H_2PO_4$, NH_4NO_3) and organic nitrogen sources (tryptone and beef extract) have effects on the production of phytase after 48 hours of incubation (Fig.8). The results showed that production was higher in media enriched with inorganic N ($NH_4H_2PO_4$) as the nitrogen source. In accordance to this result, Vohra & Satyanarayana, (2003), Mittal *et al*. (2012) reported that maximum production for *Mycelophoythora thermophile* and *Klebsiella* sp. respectively were when $NH_4H_2PO_4$ was used as the nitrogen source. Some researchers stated that inorganic N sources, such as $NH_4H_2PO_4$ (Gulati *et al*. 2007) and NH_4NO_3 (Fu *et al*. 2011), provided higher phytase production compared to organic N. Similarly, Tahir *et al*. (2010); Sreedevi & Reddy (2012) reported that the highest production was obtained on NH_4NO_3 enriched media.

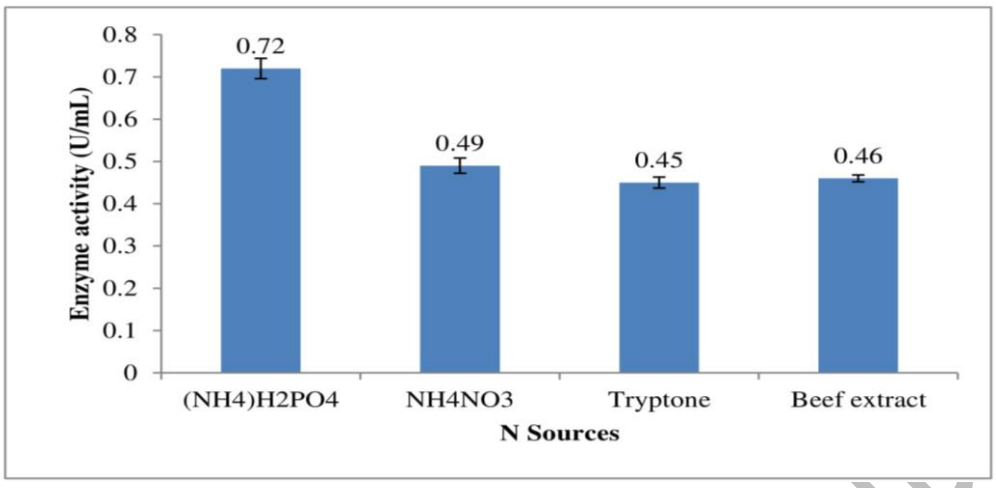

Figure 8 Effect N sources on phytase production by *Enterobacter cloacae*

CONCLUSION

There were 13 phytase producing bacteria (PPB) that were isolated from the legume rhizosphere. The highest hydrolysis efficiency (3.43) was identified as a *Enterobacter cloacae* strain B1. The maximum phytase-production of strain *E. cloacae* which was grown in liquid PSM was obtained at 48 hours of incubation. This strain produced optimum levels when lactose and $NH_4H_2PO_4$ was utilized as the carbon and nitrogen sources respectively. The optimum results of phytase activity were obtained at 30^{o}C and pH 5.0 in the physical parameters. PPB obtained in the present study can be exploited for their use as biological fertilizers to plants.

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